Subunit dynamics in *Escherichia coli* preprotein translocase

(SecY/SecE/band 1/subunit exchange/epitope tag)

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ABSTRACT   SecY, SecE, and band 1 copurify as the
SecY/E integral membrane domain of *Escherichia coli* pre-
protein translocase. To measure the *in vivo* association of these
polypeptides and assay possible exchange, plasmid-borne *secY*
and *secE* genes were placed under control of the *ara* regulon
and fused to DNA encoding the influenza hemagglutinin epi-
tope. Cells were incubated with [³⁵S]methionine, grown for a
"chase" period, and then induced with arabinose to express
epitope-tagged, nonradioactive SecY and SecE. Both the wild-
type and epitope-tagged polypeptides assembled into func-
tional, heterotrimeric SecY/E complex. However, immuno-
precipitation with antibody to the epitope tag did not cross-
precipitate radiolabeled SecY or SecE. Thus, these subunits
normally associate stably *in vivo*.

Protein secretion in *Escherichia coli* has been investigated by
both biochemical and genetic techniques (1–3). Studies of
temperature-sensitive *sec* mutants (4, 5) and suppressor *prl*
mutants (6) identified the genes essential for secretion. Bio-
chemical studies revealed that several of the encoded pro-
teins form subunits of preprotein translocase, a membrane
enzyme which catalyzes the movement of precursor proteins
across the cytoplasmic membrane (7). The enzyme consists
of an integral membrane domain, termed SecY/E, and a
peripheral membrane domain, the SecA protein. SecY/E
consists of three subunits: SecY, SecE, and band 1 (7). Both
SecY and SecE have been identified by biochemical and
genetic techniques as being required for translocation. Band
1 copurifies with SecY and SecE in detergent extracts and
can be coimmunoprecipitated with SecY and SecE by anti-
bodies to the amino terminus of SecY (7, 8). The purified
trimeric complex can be reconstituted into liposomes that, in
the presence of SecA and ATP, translocate precursor pro-
teins.

Despite the isolation of a complex containing SecY and
SecE, some studies have suggested that SecY and SecE may
not remain stably associated. Elegant genetic studies involv-
ing suppressor-directed inactivation and *sec* titration tech-
niques suggest that the SecY and SecE proteins reversibly
assemble in the membrane (9, 10). These studies are based on
strains containing protein localization mutants (*prl*) in the
SecY or SecE protein together with a leader sequence
mutation in a fusion protein (LamB–LacZ). When the mutant
fusion protein is expressed, it is recognized by the *prl* mutant
protein and "jams" the translocase. The cells stop growing
unless the wild-type *prl* allele, whose protein product does
not recognize the mutant leader sequence, is also expressed.
To explain these observations, it was postulated that the
preprotein translocase subunits associate reversibly as part
of the catalytic cycle of translocation. The suggestion that
SecY, SecE, and band 1 may not always remain stably
associated has also come from biochemical studies. Under

certain conditions, these proteins can be isolated separately.
When co-reconstituted into proteoliposomes, they can func-
tion together to support translocation (11). Each of these
studies has raised the question of whether SecY, SecE, and
band 1 are normally stably associated *in vivo*.

To test this association, we have prepared genes encoding
epitope-labeled SecY and SecE. We now report that the
SecY and SecE proteins are stably associated. Exchange of
these subunits is not detectable, even after a generation of
growth in which hundreds of translocation events have
occurred at each export site. Under certain conditions, when
SecE is epitope-labeled, there is extensive and selective
exchange of band 1.

MATERIALS AND METHODS

Plasmid Constructions. The *E. coli secY* gene in plasmid
pKY6 (5) was amplified by the polymerase chain reaction
(PCR) with primer derived from the amino terminus (5′-
ACGGAATTCACCATGTACCCATACGATGTTCCT-
GACTATGCGGGCGGCCCAGCTAAACAACCGGGATT-
AGATTTTC-3′) fused with an influenza hemagglutinin (HA)
epitope (12, 13). This also introduced an *Eco*RI site for
cloning. The epitope protein sequence, Tyr-Pro-Tyr-Asp-
Val-Pro-Asp-Tyr-Ala, was fused to the SecY sequence
through a Gly-Gly-Pro motif to aid in displaying the epitope
from the SecY protein (12). The oligonucleotide primer from
the carboxyl terminus (5′-GCCGTCGACTTATCGGCCG-
TAGCCTTTC-3′) introduced a *Sal* I site. After amplification
by *Pfu* polymerase (Stratagene), the DNA fragment was
digested with *Sal* I and *Eco*RI and ligated into pUC19 (14).
The *E. coli secE* gene in the plasmid pBAD22 (gift of J.
Beckwith, Harvard Medical School) was amplified by PCR,
introducing a *Sal* I site before the amino terminus (5′-
GGCGTCGACAGGTTGGTTTATGAGTGCGAATAC-
CGAA-3′) and a *Hind*III site after the carboxyl terminus
(5′-CAGAAGCTTTCAGAACCTCAGGCCAGTG-3′). Both
the pUC19 derivative encoding HA epitope-labeled SecY
(pHA-SecY) and the amplified *secE* fragment were digested
with *Sal* I and *Hind*III. The fragments were ligated to produce
epitope-labeled *secY* followed by wild-type *secE*. The result-
ing plasmid was digested with *Eco*RI and *Hind*III and the two
fragments containing the *sec* genes were ligated into pBAD22
(to give pHA-SecY/SecE) and transformed into *E. coli*
JM109 (14). The same cloning strategy was used to create
epitope-tagged *secE* followed by wild-type *secY* in pBAD22.
The oligonucleotide primers for epitope-tagged *secE* were
5′-ACGGAATTCACCATGTACCCATACGATGTTCCT-
GACTATGCGGGCGGCCCAAGTGCGAATACCGAAGC-
TCAAGGAA-3′ for the amino terminus and 5′-GCAGTC-
GACTCAGAACCTGAGGCCAGTG-3′ for the carboxyl ter-
minus. The oligonucleotide primers for the *secY* gene were

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5'-GCGGTCGACAGGAATAAGTAGCAGATGGCTAAA-CAACCGGGATTA-3' for the amino terminus and 5'-GCCAAGCTTTTATCGGCCGTAGCCTTTC-3' for the carboxyl terminus. In both final plasmids, the epitope-labeled gene possessed AGGAGG as the Shine–Dalgarno sequence and the wild-type genes were amplified to include their respective Shine–Dalgarno sequences. The plasmid-borne genes were fully functional, as assayed by complementation of temperature-sensitive strains.

Bacterial Growth. Bacteria were grown overnight in LB medium (15) with ampicillin at 37°C and diluted to an OD_{600} of 0.05 into 50 ml of M9 medium with thiamin (10 $\mu g/ml$), ampicillin (50 $\mu g/ml$), amino acids (40 $\mu g/ml$, without methionine), and 0.2% (wt/vol) glucose. For the subunit-exchange experiments, 2 mCi (74 MBq) of EXPRE^{35}S^{35}S protein labeling mix (NEN/DuPont) was added to a 50-ml culture (OD_{600} of 0.2) for 30 min at 37°C. The cells were centrifuged for 5 min at 3000 × g at 20°C and then were suspended in 50 ml of M9 medium containing thiamin, ampicillin, amino acids, and 0.2% (wt/vol) fructose. Methionine (50 $\mu g/ml$) was added and the cells were grown to an OD_{600} of 0.8. Arabinose (1%, wt/vol) and, where indicated with strain R0205, maltose (0.4%) were added and growth continued for 30 min. The cells were harvested by centrifugation (for 5 min at 3000 × g at 20°C), suspended in 50 mM Tris Cl, pH 7.5/10% sucrose, frozen dropwise in liquid nitrogen, and stored at −70°C.

Membrane Preparation. Membranes were prepared for immunoprecipitation by a modification of the method of Kaback (16). An aliquot of cells was thawed and EDTA (10 mM) and lysozyme (0.3 mg/ml) were added. After 8 min at 23°C, ice-cold water was added (>40 volumes) and the samples were mixed vigorously. Samples were centrifuged for 5 min at 3000 × g at 4°C to remove unbroken cells and the resulting supernatants were centrifuged for 20 min at 40,000 × g at 4°C. Pellets were suspended in 5 ml of 50 mM Tris Cl, pH 7.5/10 mM EDTA. Nucleic acids were digested by addition of 20 mM $MgCl_2$ and DNase and RNase (each at 5 $\mu g/ml$) for 5 min at 23°C. Membranes were sedimented for 20 min at 40,000 × g and suspended in 0.6 ml of 50 mM Tris Cl, pH 7.5/10 mM EDTA. Aliquots were assayed for radioactivity and protein (17). Typically 5 × 10^6 cpm of ^{35}S-labeled membranes was used for each immunoprecipitation.

Immunoprecipitations and Immunoblots. For nondenaturing immunoprecipitations, membranes were solubilized (18) by incubation on ice for 15 min with 1.25% (wt/vol) octyl β-D-glucoside in 10 mM Tris Cl, pH 8.0/25% (vol/vol) glycerol containing *E. coli* phospholipids (Avanti Polar Lipids) at 3.4 mg/ml. Samples were centrifuged for 15 min at 16,000 × g, and the supernatants were added to 300 μl of immunoprecipitation (IP) buffer (50 mM Tris Cl, pH 8.0/150 mM NaCl/1.25% octyl β-D-glucoside, 40% glycerol with *E. coli* phospholipids at 1.5 mg/ml). Antibodies were added to 100 μl of a 10% (vol/vol) suspension of protein A-Sepharose CL-4B (Pharmacia) in 2% (wt/vol) bovine serum albumin, incubated for 1 hr at 4°C with constant mixing, collected by brief centrifugation, suspended in an equal volume of IP buffer, and again collected by centrifugation. Beads were suspended in membrane extracts, the incubation was continued for 1 hr, and complexes were harvested by centrifugation (for 2 min at 4°C). The beads were twice resuspended and centrifuged, each with 1 ml of IP buffer. Proteins were eluted with 25 μl of 4% (wt/vol) SDS/160 mM TrisCl, pH 6.8/200 mM 2-mercaptoethanol/20% (vol/vol) glycerol/0.02% (wt/vol) bromophenol blue and incubated for 10 min at 37°C. The beads were removed (microcentrifuge, 2 min) and the supernatants were electrophoresed in high-Tris SDS/polyacrylamide gels (7) and analyzed by fluorography. For immunoblots, samples were transferred from SDS/polyacrylamide gels to poly(vinylidene difluoride) mem-

branes at 200 mA for 1 hr with an Idea Scientific (Corvallis, OR) apparatus. Membranes were incubated for 12 hr with a 1:500 dilution of antiserum, developed by the ECL Western detection system (Amersham), and quantified by densitometry.

Antibody Preparation. Antibody was prepared to the SecE peptide ATVAFAREARTEVRKVIWPTRQETC. Antiserum to the amino terminus of SecY has been described (19, 20). Typically, 25 μl of heat-inactivated antiserum was used per immunoprecipitation. Monoclonal antibody 12CA5 and ascites preparation were as described (12). Ascites proteins were precipitated with ammonium sulfate at 50% saturation and dialyzed versus phosphate-buffered saline. Typically 2 μl of this concentrated antibody solution (16 mg/ml) was used per immunoprecipitation.

RESULTS

To test whether SecY and SecE were stably associated *in vivo*, we fused an epitope of influenza HA to either SecY or SecE. This epitope tagging method has been used by several investigators to purify multisubunit protein complexes (12, 21). The epitope was introduced at the amino terminus of either SecY or SecE, with the wild-type gene for the other Sec protein also encoded on the same plasmid under *ara* control. Epitope-tagged SecY and SecE were each fully functional in rescuing growth of strains with conditional lethal mutations in the *secY* or *secE* genes. *E. coli* CJ107 has a temperature-sensitive SecY (22). When either the epitope-tagged or the wild-type *secY* gene was present on a plasmid (Fig. 1A, sectors II and III, respectively), cells grew at 42°C. The background vector or a *secE*-containing plasmid did not complement (sectors I and IV). *E. coli* AF29 has a cold-sensitive mutation in *secE* (23). When a wild-type or epitope-
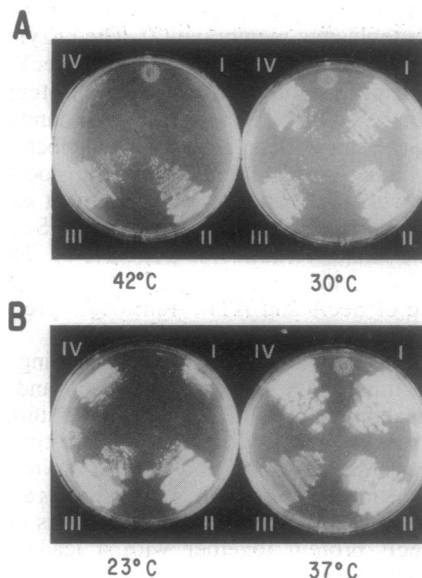

FIG. 1. Complementation of temperature-sensitive SecY and SecE strains by epitope-tagged subunits. (A) The *secY* temperature-sensitive strain CJ107, containing plasmid pHA-SecE (sectors I), pHA-SecE/SecY (sectors II) pHA-SecY/SecE (sectors III), or pBAD18 (sectors IV). The left plate was incubated at the nonpermissive temperature (42°C) and the right plate was incubated at the permissive temperature (30°C). (B) The SecE cold-sensitive strain AF29 (a gift from Ann Flowers, containing a *secE15* cold-sensitive mutation) containing pHA-SecY (sectors I), pHA-SecE/SecY (sectors II), pHA-SecY/SecE (sectors III), or pBAD18 (sectors IV). The left plate was incubated at the nonpermissive temperature (23°C) and the right plate was incubated at the permissive temperature (37°C). All plates contained agar with LB medium plus arabinose (0.02%) and ampicillin (50 $\mu g/ml$).

tagged *secE* gene was present (Fig. 1*B*, sectors II and III, respectively), the defect was complemented.

Labeling Epitope-Tagged SecY/E. To examine the epitope-tagged Sec proteins *in vivo*, we metabolically labeled cells for 30 min with a mixture of ^{35}S-labeled methionine and cysteine after induction of the plasmid-borne genes. Previous work showed that a complex of wild-type SecY, SecE, and band 1 can be isolated from detergent extracts (7). SecY undergoes an endoproteolytic event in these extracts (without loss of translocation activity; ref. 7), and the presence of the epitope tag affects the mobility of the Sec proteins in SDS/polyacrylamide gels. Immunoprecipitates with the HA antibody of membrane extracts from cells with the plasmid pHA-SecY/SecE therefore contained the HA-tagged SecY amino-terminal fragment, the SecY carboxyl-terminal fragment, band 1, and SecE (Fig. 2, lane 2). When this same extract was immunoprecipitated with antibody to the native SecY amino terminus, the amino-terminal fragments of both wild-type SecY and the HA-derivatized SecY were recovered, as well as the SecY carboxyl-terminal fragment, band 1, and SecE (lane 1). These data show that comparable amounts of tagged and wild-type SecY were being made and that both wild-type SecY and HA-derivatized SecY assemble into the SecY/E ternary complex. Furthermore, the absence of wild-type SecY(N) fragment in the anti-HA immunoprecipitates (lane 2) suggests that there is only one SecY subunit per SecY/E protein. Similar results were obtained with nondenatured immunoprecipitates of membrane extracts from cells labeled while synthesizing HA-derivatized SecE (lanes 3 and 4). HA-derivatized SecE assembled into the SecY/E complex (lane 4), which did not have wild-type SecE, suggesting that SecY/E has only one SecE subunit.

Immunoprecipitation with antibodies to SecY (Fig. 2, lane 3) confirmed that both wild-type and HA-derivatized SecE were being synthesized in comparable amounts and were assembling into complex with SecY and band 1. Immunoblot analysis with antibody to SecY and SecE (Table 1) confirmed that SecY, though labile when overproduced in isolation, was stably accumulating as overproduced with SecE, whether the epitope tag was on SecY or on SecE, as reported (24, 25).

Examination of SecY and SecE Dynamics. To assay subunit exchange between old and new SecY/E complexes, bacteria containing the plasmid for epitope-tagged SecY and wild-type SecE were labeled with [^{35}S]methionine/[^{35}S]cysteine for 30 min under noninducing conditions in the early logarithmic phase of growth. During this labeling period, chromosomally encoded SecY and SecE were synthesized but little epitope-tagged SecY was expressed. The labeled amino acids were removed and excess unlabeled amino acids were added. After

Table 1. SecY and SecE content of inner membrane vesicles

Plasmid	Arabinose	Relative amount			
		Wild-type SecY	HA-SecY	Wild-type SecE	HA-SecE
pHA-SecE/ SecY	−	1	0	1	0
	+	1.8	0	0.51	0.82
pHA-SecY/ SecE	−	1	0	1	0
	+	0.84	0.56	1.5	0

Where indicated JM109 cells containing the indicated plasmid and at an OD$_{600}$ of 0.5 were induced with arabinose for 30 min, then harvested and analyzed by SDS/PAGE. The content of SecY, SecE, and their HA derivatives was determined by quantitative immunoblot.

continued growth, the culture was divided and expression of unlabeled, epitope-tagged SecY and wild-type SecE was induced in one portion with arabinose. Cells were harvested, membrane extracts were prepared, and SecY/E was immunoprecipitated by antibody to either the amino terminus of SecY or to the influenza HA epitope. Samples were analyzed by SDS/PAGE and fluorography. If there was free exchange of SecY and SecE subunits between enzyme complexes, then the unlabeled, epitope-tagged SecY should have been associated with radioactive SecE. This was not the case. ^{35}S-labeled SecY, SecE, and band 1 were readily detected in a complex by immunoprecipitation with antibodies to the SecY amino terminus (Fig. 3, lanes 5 and 7). For the culture where, after ^{35}S-free chase period, arabinose was added to induce expression of the epitope-tagged SecY and SecE, the immunoprecipitate with anti-HA antiserum (lane 8) was similar to that seen without arabinose induction (lane 6). Since SecE has only three methionines, greater sensitivity is afforded by the plasmid construction in which SecE is epitope-tagged. When the experiment was repeated with epitope-tagged SecE, no labeled SecY was detected in the anti-epitope immunoprecipitates (Fig. 3, lane 4). Radioactive SecY was present and stably associated in SecY/E complexes (lanes 1 and 3) but did not exchange to become associated with the epitope-tagged SecE protein. The only radioactive subunit seen coimmunoprecipitating with the tagged SecE was band 1 (lane 4); the significance of this observation is unclear. We conclude that the SecY and SecE subunits are stably associated *in vivo* and do not freely exchange.

Subunit Dynamics in an Export Mutant Strain. Bieker-Brady and Silhavy (10) have suggested that SecY and SecE might dissociate and reassociate as part of their catalytic cycle, whereas our studies suggest that they remain associated. To determine whether this reflects a strain difference,
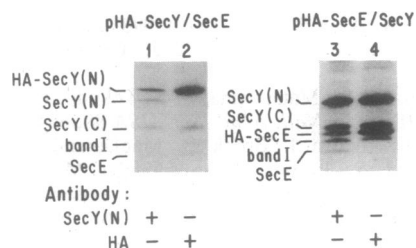

FIG. 2. Expression of plasmid-borne and epitope-labeled SecY and SecE. Bacteria (*E. coli* JM109) were grown as described in *Materials and Methods* except that 1% arabinose was added when the culture reached an OD$_{600}$ of 0.5, followed by [^{35}S]methionine/cysteine. After 30 min, cells were harvested, frozen, and processed for immunoprecipitation, electrophoresis, and fluorography. Lanes 1 and 2, cells expressing epitope-tagged SecY and wild-type SecE; lanes 3 and 4, cells expressing epitope-tagged SecE and wild-type SecY. Antibodies used for immunoprecipitation are indicated below the lanes. N and C in parentheses signify amino-terminal and carboxyl-terminal fragments, respectively.
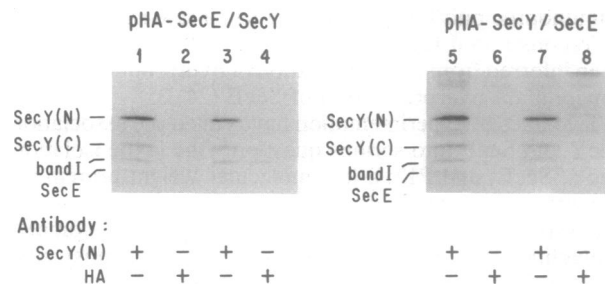

FIG. 3. *In vivo* association of SecY/E subunits. Cells (*E. coli* JM109) bore plasmid pHA-SecE/SecY (lanes 1–4) or pHA-SecY/SecE (lanes 5–8). After radiolabeling, cells were either left uninduced for expression of plasmid-borne genes (lanes 1, 2, 5, and 6) or were induced (lanes 3, 4, 7, and 8). Membrane extracts were immunoprecipitated with either 12CA5 monoclonal antibody (anti-epitope) or SecY amino-terminal antibodies as indicated.
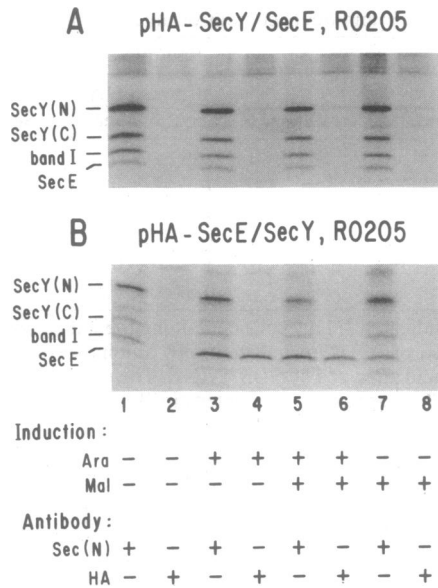
A   pHA-SecY/SecE, R0205

SecY(N) —
SecY(C) —
band I —
SecE —

B   pHA-SecE/SecY, R0205

SecY(N) —
SecY(C) —
band I —
SecE —

1   2   3   4   5   6   7   8

Induction :

Ara   −   −   +   +   +   +   −   −
Mal   −   −   −   −   +   +   +   +

Antibody :

Sec(N)   +   −   +   −   +   −   +   −
HA   −   +   −   +   −   +   −   +

FIG. 4. SecY/E subunit dynamics in *E. coli* R0205. Fluorographs of immunoprecipitations of cells with either pHA-SecY/SecE (*A*) or pHA-SecE/SecY (*B*). Bacteria were grown, labeled, induced with the indicated sugars (Ara, arabinose; Mal, maltose), and analyzed as described in *Materials and Methods*.

we transformed *E. coli* R0205 (MC4100 *prlA4, lamB17D–lacZ42-1*) with plasmids expressing SecY and SecE, with the HA epitope label on either SecY (Fig. 4*A*) or SecE (Fig. 4*B*). After metabolic labeling with [^{35}S]methionine/[^{35}S]cysteine, followed by a chase period as above, the cells were induced with combinations of arabinose and maltose to express the plasmid-borne genes or the LamB17D–LacZ42-1 fusion protein, respectively. Immunoprecipitation with antibodies to SecY showed that the SecY/E complexes remained firmly associated in all cases (Fig. 4, odd-numbered lanes). Immunoprecipitation with antibodies to the HA epitope (even-numbered lanes) showed exchange of only band 1 and, as for the wild-type JM109 host, this was seen only when HA-SecE was induced.

DISCUSSION

Several previous studies have indicated that SecY, SecE, and band 1 are associated. They copurify as a complex which can be reconstituted into proteoliposomes for translocation (7). Furthermore, antibody to the amino terminus of SecY can, under nondenaturing conditions, cross-immunoprecipitate SecE and band 1 from detergent extracts. This complex is labile in detergent solution and, during incubation, the cross-immunoprecipitation is lost at the same rate as the ability to be reconstituted for translocation (8). Association has also been inferred from the stabilization of overproduced SecY by concomitant overproduction of SecE (24, 25).

Two lines of experimentation have called the association of SecY and SecE into serious question. One is the isolation of SecY, SecE, and P12, a low molecular weight protein (with properties similar to band 1), as separate polypeptides which can be reconstituted together to form translocation-competent proteoliposomes (11). Though these data are most simply explained as a reconstitution of active SecY/E from its subunits, they serve to raise the question of whether the association seen in detergent extracts is an *in vitro* artifact. A second line of experiments is the elegant studies of Bieker-Brady and Silhavy (9, 10), using techniques of secretion-directed inactivation and Sec titration. In these experiments, cell growth was blocked by the jamming of translocation sites during the synthesis of LamB–LacZ fusion proteins. Mutations in the

leader sequence of the LamB moiety of these fusions relieved the lethality, presumably because the protein was no longer recognized for export. Lethality could, however, be restored by the selective introduction of suppressor *prl* alleles in the SecA, SecY, or SecE genes. These studies suggest that the proteins encoded by *sec* genes can be separately titrated, and that they assemble in a specific order during each catalytic cycle of translocation. While we have not reconciled these studies with those reported here, the molecular structure of the "jammed" intermediates and the effects of this jamming on translocase are not known.

Our studies establish that SecY, SecE, and band 1 are stably associated with one another *in vivo*, as apparent subunit exchange would be seen if the association had first occurred in the detergent extracts. This association is stable during growth times which correspond to hundreds of catalytic turnovers. Induction of fusion protein in suppressor strains does not promote general subunit exchange (Fig. 4). We do, however, see apparent exchange of band 1 into a complex with HA-tagged SecE. The significance of this finding must await further studies of band 1. The recent finding that band 1 and protein P12 are identical (K. Douville, M.R.L., L. Brundage, K.-i. Nishiyama, H. Tokuda, S. Mizushima, and W.T.W., unpublished work) will make this possible.

Other multiprotein systems exhibit subunit exchange (26–31). However, only a few oligomeric membrane proteins have been examined for the dynamics of their subunit association (32, 33). We are not aware of other studies of the *in vivo* exchange rates of subunits between assembled heterooligomeric enzyme molecules. Epitope tagging provides a means to explore subunit exchange in living cells.

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